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The behavioural effects of supplementing diets with synthetic and naturally sourced astaxanthin in an ornamental fish (*Puntius titteya*).

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Abstract

Carotenoids are routinely incorporated into ornamental fish diets with the aim of enhancing companion fish colouration which may concomitantly affect fish behaviour. Previously, colour enhancement has typically been achieved using synthetic carotenoids, however, there is now growing public demand for food additives such as carotenoids to be derived from natural sources, which can be acquired from microalgae and cyanobacteria. There has been very little research into whether natural carotenoids alter fish behaviour in a similar way to synthetic carotenoids; the present study aimed to determine whether behavioural changes typically associated with increased carotenoid consumption differed according to carotenoid source in the cherry barb (*Puntius titteya*). Cherry barbs were fed one of four diets (carotenoid-free, 20 ppm synthetic astaxanthin (AX) sourced from Carophyll

pink®, 20 or 40 ppm of natural-AX sourced from Panaferd) over a 12 week period and then observed for colour changes, mate-choice and aggressive behaviours. The diets containing 20 ppm synthetic-AX and natural-AX enhanced male red colouration of the anal fin and anterior dorsal area, *via* a reduction in hue, in comparison to the carotenoid-free control diet whereas only the 20 ppm natural-AX altered the hue of female colour. In the mate choice trials, males spent more time with females fed the 20ppm synthetic-AX and 40ppm natural-AX compared with the carotenoid-free control and 20 ppm natural AX. Experiments conducted under red-blocking and UV blocking conditions demonstrated an effect of red colouration and ultraviolet reflectance on mate discrimination. Interestingly, males fed both the synthetic and natural AX diets reduced aggressive interactions with a mirror image, even though they displayed enhanced red colouration, which is often used by fish as a signal of increased competitive ability. In conclusion, source of dietary AX affected the behaviour of cherry barbs, to the extent that synthetic AX exerted a stronger effect on mate-choice behaviour under full spectrum lighting in comparison to a similar concentration of natural AX. This therefore demonstrates that the behaviour of companion fish can be influenced by the source of carotenoids within their food.

Keywords: Carotenoids, mate-choice behaviour, mirror-image tests, cherry barbs, ornamental fish.

1. Introduction

There are numerous nervous (Amiri and Shaheen, 2012), endocrine (Leclercq *et al.*, 2010) and dietary (Harpaz and Padowicz, 2007) processes which can affect the colouration of teleost fishes, and

54 therefore the transfer of information within colour-based visual signals
55 (Evans and Norris, 1996; Baron *et al.*, 2008). Carotenoid pigments are
56 an important dietary requirement; their properties as antioxidant
57 compounds (Sies and Stahl, 1995) and ability to alter colouration have
58 been well documented in teleost fish as well as many other taxonomic
59 groups (McGraw *et al.*, 2002; Blount, 2004). Baron *et al.* (2008)
60 demonstrated that alterations in colour through carotenoid
61 consumption can have subsequent effects for colour based behaviours.
62 Female flame-red dwarf gourami (*Colisa lalia*) preferentially
63 associated with male fish exhibiting lighter colouration after Lucantin
64 Pink consumption (Baron *et al.*, 2008). Evans and Norris (1996) found
65 that male fire-mouth cichlids (*Cichlasoma meeki*) fed with increased
66 carotenoids were more successful in aggressive interactions than
67 opponents fed with a reduced amount of carotenoids. This difference
68 was not seen when the experiments were conducted under green
69 lighting that prevented fish from discriminating between red colours.
70 Hence the difference in success of individual males was directly
71 attributed to the effects of carotenoids on the red colour patches used
72 for signalling, and not to any other factor such as mass or size.

73 In addition to assessing the effects of carotenoids on the use
74 of colour signals within the human visual spectrum, it must be noted
75 that a number of fish species are sensitive to ultraviolet light: UVA
76 wavelengths specifically, with a peak absorption of 360 nm in teleost
77 cone cells (Losey *et al.*, 1999). Guppies (*Poecilia reticulata*) and
78 three-spined sticklebacks (*Gasterosteus aculeatus*) both use ultraviolet
79 reflectance during mate choice assessment (Kodric-Brown and
80 Johnson, 2002; Rick and Bakker 2006; Rick and Bakker, 2008a).
81 Dietary carotenoids can affect ultraviolet reflectance either directly (by

interacting with ultraviolet light) or indirectly (by affecting the presence of other pigments) (Kodric-Brown & Johnson, 2002). When assessing the impacts of a carotenoid diet on colour based behaviours, it is therefore important to consider the visual capacity of the species involved (Bennett and Cuthill, 1994) and whether UV-reflectance-based behaviours are used.

Generally, reproductive output is limited by female capacity to breed, which therefore drives male competition for access to females (Sargent *et al*., 1986). As reproductive investment is generally reduced for males in comparison to females, mate choice studies have predominantly used females as focal individuals and assessed female mate choice. However, this does not necessarily mean discrimination between potential mates solely occurs by females; male fish are also selective in their choice of mating partners. For instance, male Pacific blue-eye (*Pseudomugil signifier*) fish discriminate between females based on size and preferentially associate with larger females providing there is no additional cost to them (Wong & Jennions, 2003). Additionally, male two-spotted gobies (*Gobiusculus flavescens*) associate with potential mates based on assessments of female coloured ornaments (Amundsen & Forsgren, 2001). Preliminary behavioural observations of cherry barbs revealed female fish to be shyer than males, with male fish constantly attempting to court females. Subsequently, a mate choice model was established which used male fish as the focal fish which enabled assessment of both male and female mate choice.

Carotenoid consumption alters the expression of certain behaviours to correlate with resource-holding potential, however, carotenoid absorption and storage in tissues is dependent upon its

chemical form as well as the ability of the fish to convert it into other carotenoids, which differs according to taxonomic grouping. In recent years there has been an increase in consumer demand for the use of products and raw materials which are naturally derived with less dependence on synthetic or highly processed goods. Whether natural or synthetic additives exhibit different effects due to bioavailability is contested. For instance, vitamin C as an additive is synthetically produced with an identical chemical structure to its naturally occurring counterpart (Carr & Vissers, 2013). In human experiments natural and synthetic vitamin C are equally bioavailable, however in animal studies there is greater variation in natural *versus* synthetic bioavailability dependent upon the animal model used (Vissers *et al*., 2011; Carr & Vissers, 2013). Naturally produced supplements are often synthesised in conjunction with other compounds which are thought to influence bioavailability, an example being the interaction between flavonoids and vitamin C affecting uptake (Song *et* al., 2002; Vissers *et* al., 2011). Despite variable bioavailability in animal models, the trend for naturally derived additives has moved from human foods into those fed to our companion animals and wherever possible natural colourants, preservatives and flavourings are utilised. For fish, ingredients such as natural colourants, particularly those which help to enhance the colouration of fish and may provide additional health benefits, such as carotenoids have been focussed on (Sinha & Asimi, 2007; Yanar *et al*., 2008). In this study, colour expression and colour-associated behaviours were assessed in cherry barbs fed one of two astaxanthin-based flake diets, Carophyll-pink and Panaferd. Carophyll-pink is a synthetically produced astaxanthin (AX) whereas Panaferd is sourced from a novel natural fermentation method from

Paracoccus carotinifaciens. The main carotenoid component of Panaferd is astaxanthin, however, as it is naturally occurring it also contains several other carotenoids in lower quantities, it is not known whether the presence of additional carotenoids or other naturally occurring compounds alter the bioavailability of astaxanthin. Panaferd, a new ingredient proposed for commercial diets, was tested at two concentrations (20 and 40 ppm) alongside Carophyll-pink (20 ppm) to determine the effects of consumption of astaxanthin produced from a natural source. A number of different parameters were measured to assess the effects of diets on male and female cherry barbs, *Puntius titteya*. These included changes to mate choice and competitive ability, as well as changes in colour.

2.1 Methods

Cherry barbs were sourced from a local pet store and held in high density stock tanks until experiments began (dissolved oxygen 94.1% ± 0.8%; pH 7.39 ± 0.04; temperature 28.0˚C ± 0.1˚C; light:dark period 12:12; all values ± SEM). Each diet treatment (see below for diet details) consisted of six replicate tanks, with four males and four females placed in each tank. In three tanks of each diet treatment, barriers physically and visually separated sexes (n= 24 fish per diet treatment: 12 male, 12 female); of these groups of fish, three males and three females from each tank were selected for mate-choice trials (n=18 fish per diet treatments: 9 male, 9 female). Physical separation of the sexes within these tanks was used to prevent fish from exhibiting preferences during mate choice trials according to prior social encounters. The remaining three tanks of each diet treatment contained fish in mixed sex groups. All six tank replicates were included in

colour change analyses (n=48 fish per diet treatment: 24 male, 24 female).

The four diets were supplied by WALTHAM, Mars (http://www.waltham.com/), a negative control containing no pigments, a 20 ppm Carophyll-pink positive control and a 20 ppm and 40 ppm novel diet containing Panaferd-AX. Nutritional content of the diets is given in Table 1.

2.1. HPLC analyses

Carotenoid content of flake diets was determined using high performance liquid chromatography. A sample (3.0 g) of each diet was used for carotenoid determination. As carotenoids from the Panaferd source are produced by fermentation of bacteria, different extraction solvents were required to remove cell walls, as opposed to those from control and synthetic astaxanthin diet treatments. Once extracted, the samples were ultimately processed through HPLC in the same manner. Flake samples from negative and synthetic diet treatments were shaken with 0.5 ml Protex 6L, 100 mg butylated hydroxytoluene (BHT) and 6 ml D.I. water and sonicated at 50°C for 30 min. 40 ml of ethanol was added to the suspension, shaken and 50 ml of dichloromethane added. The mixture was allowed to cool to room temperature in the dark for 2 hours. Extracts were then purified by open column chromatography on silica gel. Carotenoids were then eluted from the silica gel with 5 ml iso hexane : diethyl ether (1:1) and evaporated under nitrogen. Carotenoids were reconstituted in 1 ml of iso hexane : acetone (82:18). Flake samples of Panaferd diets were sonicated with 2.5 D.I. water at 60°C then shaken with 5 ml of tetrahydrofuran (THF) : methanol (20:1) for 5 min. Solutions were then centrifuged at 1300 rpm for 10

193 min with 10 ml of isohexane. A 5 ml aliquot was then dried under
194 nitrogen and reconstituted in 5 ml of isohexane. The HPLC (Dionex
195 Ultimate 3000) used an autosampler with an injection volume of 33 µl.
196 The mobile phase used was iso-hexane : acetone : iso-propanol
197 (82:16:2) at 25°C with flow rate at 1.5 ml min^{-1}. The column used was
198 a Luna 3 µm silica analytical column (length: 100 mm, diameter: 4.6
199 mm), carotenoid amounts were quantified at 474 nm. Carotenoid
200 contents are expressed as mg kg^{-1} in Table 1.

201 2.2. Colour analysis

202 At the start of the experiment, male and female cherry barbs
203 were lightly anaesthetised using MS-222 (0.08 g l^{-1}) according to
204 Sloman *et al.* (2003) and held in a petri dish containing enough water
205 to cover the body of the fish. The right hand lateral side of each fish
206 was photographed using a Canon EOS 60D dSLR. The following
207 camera settings were used according to the recommendations of
208 Stevens *et al.*, (2007); manual white balance, manual focus, relative
209 aperture f/8, shutter speed 1/40s, ISO 320. The camera was mounted
210 on a tripod at a set distance above the fish, a spotlight was used to
211 provide constant illumination. All fish recovered from anaesthesia
212 without any observable adverse effects. Different anaesthesia methods
213 have previously been shown to affect spectral reflectance patterns
214 (Gray *et al*., 2011), one method (MS-222) was therefore used across
215 all colour measurements. Following this, fish were fed their respective
216 diets for a period of 12 weeks, fish were fed to satiation to ensure food
217 intake was even within groups and not controlled by the formation of
218 social hierarchies. Nutritional differences between diets were minimal
219 (Table 1), thus it was assumed that diets did not provoke differences
220 in appetite and that carotenoid intake was maintained at intended levels

221 *via* relative concentrations within diets. No underweight or overweight
222 fish were observed during feeding trials. Fish were then photographed
223 again according to the methods previously outlined, and behavioural
224 trials then took place.

225 Images were calibrated to a full colour standard (x-rite
226 ColorChecker Passport http://www.xrite.com/home.aspx:
227 ColorChecker Passport v1.0.1) and graphical software (Photoshop
228 CS5) was used to isolate specific areas of an image to allow for colour
229 analyses in various body areas (Fig. 1). These areas consisted of the
230 whole body which was then broken down into caudal fin, anal fin and
231 anterior dorsal areas. Images were then analysed using two different
232 MATLAB codes.

233 2.2.1. %Red and %Yellow calculations

234 MATLAB analysed the percentage of pixels within an image
235 that were either 'red' or 'yellow' based on predefined colour
236 parameters. The red and yellow parameters were adapted from Maan
237 *et al*. (2010) in which pixels would be identified as red if the hue was
238 within 0-26 or 232-255 of the 0-255 RGB hue scale, yellow was
239 defined as hues of 27-45. If pixels fell within these hue ranges they
240 were then counted as red or yellow providing they met saturation
241 criteria of 40-97 (Fig. 1). MATLAB analysed colouration within the
242 HSV (hue, saturation and value) scale and not the RGB (red, green and
243 blue) scale, which runs from 0-1.0 rather than 0-255. Red and yellow
244 parameters were adapted to fit within the HSV scale. MATLAB
245 therefore identified red and yellow pixels based on the following
246 criteria:

247 Red: Hue = 0-0.0833 or 0.9167-1.0, Saturation = 0.40-0.97

248 Yellow: Hue = 0.0833-0.2499, Saturation = 0.40-0.97

249 2.2.2. Hue distribution

250 MATLAB also identified the distribution of hue within an
251 image as an indication of overall colouration. The hue of each pixel
252 was analysed and a histogram generated, the peak of which represents
253 the most prevalent hue. Hue was plotted against normalised pixel
254 count in order to standardise different numbers of pixels per image.
255 This method also works within the HSV scale, therefore the hue of the
256 peak has the same colour parameters set out within the %Red and
257 %Yellow calculations.

258 2.3. Behavioural assays

259 Mate choice behaviour was assessed by allowing an individual
260 male visual access to four females in a purpose built mate choice
261 chamber. Each male was allowed to assess four females each from
262 different diet treatments under three different scenarios: 1) under full
263 spectrum lighting, 2) with red reflectance blocked using green lighting
264 and 3) with ultraviolet reflectance blocked using UV filters.

265 The three mate choice scenarios were run simultaneously.
266 Each of the three lighting scenarios contained four randomly chosen
267 female fish, each from a different diet treatment (n=9 females per diet
268 treatment). Three males from each diet replicate were randomly
269 divided amongst the three lighting conditions and rotated until each
270 male experienced all three conditions successively but in a different
271 order. This was done for males of all four diet treatments. The order in
272 which males completed mate choice scenarios was randomised to
273 negate effects of prior experience. Thus, in total nine male fish per diet
274 treatment (all replicates included) participated in a series of three mate
275 choice trials (n=9). As discussed in the introduction, enhanced red
276 colouration is used as a measure of attractiveness, therefore, in theory

277 female cherry barbs should discriminate between potential mates more
278 than males. However, in preliminary observations, male cherry barbs
279 were found to be bolder than females and female fish did not make
280 appropriate focal subjects. As males were bolder, they acclimated to
281 the mate choice chambers rapidly and began associating with
282 separated females. The experimental set up also allowed female
283 motivation to be analysed by assessing their interaction with males
284 when the male was visible.

285 At the start of the choice trials, male fish were contained
286 within a clear start box at the centre of the mate choice chamber, from
287 which all females were visible, for 10 minutes to allow acclimation to
288 the mate choice chamber. After this acclimation period, males were
289 released from the start box and allowed to explore the mate choice
290 chamber and assess females for 20 minutes while being digitally
291 recorded from above. The resulting video footage was then analysed
292 using JWatcher (http://www.jwatcher.ucla.edu/) to determine the
293 proportion of time spent associated with each female. Time spent
294 associated with a female was determined as when the male was within
295 a proximity of 5 cm from the dividing partition separating the sexes.

296 Male fish from each diet treatment were also subjected to
297 mirror-image tests (n= 9 fish per diet treatment). Males were held in
298 isolation in 5 l tanks in which there was a covered mirror at one end.
299 After 20 h within the tank, the mirror was uncovered for 10 min after
300 which the mirror was recovered for a further hour. This was done to
301 allow the fish to acclimate to the action of uncovering the mirror
302 (Sloman, 2010). The mirror was then uncovered and the number of
303 aggressive interactions fish made with the mirror, defined as bites or

lateral displays, was recorded for 1 h. The number of aggressive interactions per minute was then calculated.

2.4. Statistical analyses

All data were tested for normality by assessment of residual plots and using Kolmogorov-Smirnoff and Levene's test for homogeneity of variance. Data reported in percentage were arc-sin transformed prior to analysis. Male and female colouration data were analysed separately using one-way ANOVAs with diet treatment as a fixed factor and tank replicate as a random factor. Tank replicate was used as a random factor to take into account the within and between tank variability. Mate association data were analysed using a four way ANOVA, with female diet, male diet, lighting conditions and tank replicate as fixed factors to examine differences in behaviour between mate-choice trials held under different lighting conditions. Male fish were not individually identifiable between mate-choice trials held under different lighting conditions, thus, a random effect within a mixed model could not be used. Further analysis examined each lighting condition individually to determine differences within lighting conditions. Mirror-image interactions per minute were analysed using a one-way ANOVA with diet as a fixed factor and tank replicate as a random factor. Where significant overall effects were found, Tukey's HSD was used for post-hoc testing to identify differences between treatments, using the 5% significance level.

3. Results

3.1. Colouration

Diet treatment significantly affected the hue of male fish in two isolated areas; the anal fin and the anterior dorsal areas (Table 2:

one-way ANOVA: anal fin $F_{3,15.82}$=3.22, P=0.05; anterior dorsal area $F_{3,16.43}$=3.67, P=0.03), although Tukey's post hoc testing could not identify specific differences between treatments at the 5% level. There was no difference in the percentage change of red or yellow pixels within male or female fish images as a result of diet treatment (data not shown).

3.2. Behaviour

When mate choice trials were considered across all lighting treatments, the amount of time male fish spent with females was affected by female diet treatment (Fig. 2: four-way ANOVA: Diet: $F_{3,288}$=6.059, P<0.001), but not affected by male diet treatment (Fig. 2: four-way ANOVA: $F_{3,288}$=1.149, P=0.330). As expected, there was a significant interaction between female diet treatments and lighting conditions (four-way ANOVA: $F_{6,288}$=37.776, P<0.001) and so each of the lighting conditions was analysed separately. Female diet affected male association within each of the lighting conditions (Fig. 2: two-way ANOVA: full colour lighting: $F_{3,96}$=30.876, P<0.001; Red blocked: $F_{3,96}$=55.356, P<0.001; UV blocked: $F_{3,96}$=8.602, P<0.001). Under full colour lighting, male fish spent a significantly greater amount of time with females fed the 20ppm synthetic-AX and 40ppm natural-AX (Fig. 2). This differed to mate choice trials which were conducted under red-blocking and UV blocking conditions in which males spent the greatest time with females fed the negative control and 20 ppm natural-AX respectively (Fig. 2).

In mirror image tests, male fish fed the negative control diet were significantly more aggressive in comparison to fish fed any other carotenoid diet treatments (Fig 3: one-way ANOVA: Diet: $F_{3,31}$=14.51, P<0.003).

4. Discussion

When carotenoids are incorporated into ornamental fish diets with the aim to enhance colouration and welfare, appropriate research should be carried out to determine how this might affect colour-based behaviours. In the present study, carotenoid consumption significantly changed mate choice and competitive behaviours, both of which are likely to be influenced by colour based signals in cherry barbs.

Colour changes were expected to be more apparent between diet treatments but were observed only within hue changes in isolated areas of the male body, however, there were still substantial effects to colour-associated behaviours due to carotenoid consumption. Male fish were not individually identifiable between lighting treatments, thus, analysis of all lighting conditions together to determine differences between lighting conditions resulted in pseudoreplication. Therefore, the interpretation of behavioural differences between lighting conditions may be limited. However, to remove this pseudoreplication each lighting condition was analysed separately to determine behavioural differences within each lighting condition. It was found that male cherry barbs spent the greatest amount of time with females that were fed the 20 ppm synthetic-AX and the 40 ppm natural-AX diets, when mate choice trials were conducted under full colour spectrum lighting (Fig. 2). There was no difference in male association with females fed the 20 ppm natural-AX diet compared to

those fed the carotenoid free negative control, indicating that males preferred females fed either a synthetic astaxanthin or a comparatively high concentration of natural astaxanthin. Therefore, astaxanthin source may affect mate-choice behaviour, whereby 20 ppm of synthetic carotenoids was sufficient to induce a male mate-choice preference similar to that of 40 ppm of naturally sourced astaxanthin.

To explore the effects of red colouration further, mate choice trials were repeated under green lighting which has been used previously in similar studies to block red colouration (Evans and Norris, 1996). Results should then be causally related to the effects of carotenoids on red colouration and disassociated from other potential physiological factors which could influence mate choice assessment. However, dependent upon the visual assessment capabilities of male cherry barbs, green lighting may still allow for the assessment of other physiological factors such as UV reflectance. Under green lighting, male fish spent the most time associating with females from the carotenoid-free diet. This suggests that male fish were indeed using differences in red colouration to discriminate between females in the full lighting condition. However, it is not completely clear why under green lighting, male cherry barbs particularly associated with those females fed the carotenoid-free diet. It is possible that if there was less red colouration on these females due to lack of carotenoids in their diet, that their natural colouration would have been the least affected by green lighting and therefore they appeared the most natural of a selection of fish.

Other aspects of physiology can be used as social signals. For instance, ultraviolet reflection has been shown to enhance male attractiveness to females in guppies, where females will preferentially

associate with a male reflecting ultraviolet light when presented with two carotenoid matched males (Kodric-Brown and Johnson, 2002). Indeed, Rick and Bakker (2008b), went further in selectively excluding certain wavelengths from stickleback discrimination trials. It was found that UV wavelengths carried as much information as a signal as the long, red, wavelengths did and that removal of UV reflectance reduced attractiveness. The importance of UV signalling has only been realised recently and has been suggested to act as a private communication channel (Rick and Bakker, 2008a). The short wavelength of ultraviolet light is scattered easily in water, meaning that ultraviolet signalling is only effective in close proximities allowing information to be conveyed to intended individuals whilst not making the organism more detectable by predators. This was confirmed by Cummings *et al*. (2003) showing ultraviolet reflectance enhanced northern swordtail (*Xiphophorus nigrensis*) attractiveness to mates but not to predators. This study also established that the use of ultraviolet reflectance by northern swordtails was more prevalent in populations with greater predation pressures. The Mexican tetra (*Astyanax mexicanus*) is the natural predator of northern swordtails and is less sensitive to ultraviolet wavelengths enabling swordtails to communicate effectively while staying discreet which establishes an evolutionary basis and selection pressure for the use of ultraviolet signalling.

Mate choice trials were therefore also conducted under ultraviolet reflectance blocking conditions so that any differentiation from mate choice results under full colour spectrum lighting could be attributed to a mate choice assessment that incorporates ultraviolet information. Under UV blocking conditions, males spent the least

amount of time with females fed the carotenoid-free diet, as they did under full colour spectrum lighting. However, there were differences in mate preference for females fed the three carotenoid diets. Under full spectrum lighting, male fish spent the greatest amount of time with females fed the 20 ppm synthetic-AX and 40 ppm natural-AX diet; under UV blocking conditions males spent the greatest amount of time associating with females fed the 20 ppm natural-AX diet (Fig. 2). As demonstrated by the flake analysis (Table 1), there was a greater amount of total carotenoids in the 20 ppm natural-AX diet than the 20 ppm synthetic-AX, however, the concentration of astaxanthin was similar between these two diets. This change in association suggests that male cherry barbs may utilise ultraviolet reflection in discriminating between mates and that naturally produced astaxanthin may modify ultraviolet reflectance differentially to synthetic astaxanthin. It is unclear why this effect was seen in 20 ppm natural-AX but not at a higher concentration of 40 ppm natural-AX; there may be effects of carotenoid source and concentration on ultraviolet reflectance and how conspecifics perceive this.

Female mate choice allows an individual to pick the most sexually fit male, often through visual signals including carotenoid-based red colouration (Maan *et al.*, 2006). Kodric-Brown (1988) found male guppies with enhanced red/orange colour morphology, due to increased carotenoid consumption, had a greater mating success rate due to female preference. However, it was found that male cherry barb association with females was not influenced by the diet treatment the male fish was fed, thus, females were not interacting with males in a way to increase male motivation. This may further indicate that in cherry barbs female selection of mates is weaker than male selectivity,

467 however, further experiments in which the female is the focal animal
468 would be required to confirm this.

469 Colour morphology can also be used to assess social status and
470 competitive ability of conspecifics. For example, salmonid species are
471 known to darken the colour of their skin and sclera to signal
472 subordination to opponents, which informs opponents of defeat and
473 reduces the subsequent aggression towards subordinates (O'Connor *et*
474 *al*., 1999). Similarly, carotenoid-based red colouration has been shown
475 to increase aggression of fire mouth cichlids (*Cichlasoma meeki*)
476 (Evans and Norris 1996). Carotenoids are a limited resource, thus
477 individuals with higher carotenoid consumption are likely to have
478 higher resource holding potential and are able to compete effectively
479 enough to gain access to limited resources (Evans and Norris 1996,
480 Briffa and Sneddon, 2007). The red colouration, therefore, acts as a
481 signal to opponents informing them of their competitive ability and is
482 considered an honest signal as it cannot be replicated by other means
483 (Olsen and Owens, 1988). Within the current study, male fish were
484 held in isolation and their behaviour in response to a mirror image was
485 recorded. Males that were fed carotenoids were less aggressive than
486 males fed the carotenoid-free diet (Fig. 3). As fish respond to their
487 mirror image as if it was another individual, it is possible that the
488 carotenoid fed fish perceived their reflection to be an opponent with a
489 high resource holding potential inferred from assessing colour
490 morphology. This may reduce aggression as fish may perceive their
491 chance of winning a contest to be low. Conversely, male fish fed the
492 carotenoid-free diet may have made an assessment of their 'opponents'
493 colour morphology and may have considered their chances of winning
494 a contest to be greater than those fed carotenoids. It is again possible

that ultraviolet reflectance is used in agonistic signalling. For a fish to physically attack its mirror image it has to be in close proximity to it, which would also allow for assessment of information garnered through ultraviolet reflectance. Further experimentation could be done to block the ultraviolet reflectance within mirror image trials to ascertain whether this private channel of communication is utilised in agonistic bouts.

It was expected that, regardless of whether the novel, natural astaxanthin diet altered colour morphology, there would be an observable colour change between fish fed the carotenoid-free and synthetic-AX controls. However, observed colour changes were only minimal i.e., only occurring in specific body areas. Therefore, it may be possible that more extensive colour changes in cherry barbs are dependent on a different carotenoid, a different type of pigment (i.e. flavonoids, pteridines or pyridines) or there may have been digestibility and absorption factors which prevented more pronounced colour changes i.e. astaxanthin esterification, solubility and diet lipid content or carotenoid conversion (Bories *et al*., 2007, Guillaume *et al*., 2001, White *et al*., 2002). There may also have been an effect of using adult fish within this study, for instance, if the fish have consumed carotenoids during growth from juveniles they may have already saturated the amount of pigments within the skin prior to feeding trials. To counteract this further experiments could use fish at earlier life stages or fade the colouration of adults by feeding all fish negative controls prior to being fed trial diets. This study examined colouration from a human perspective, measuring colour within the visible range of humans, and therefore does not account for the fish's own

perception of colouration. Thus, changes to colour out-with the red and yellow colour space cannot be ruled out.

In conclusion, based on behavioural trials it appears that there were changes in red colouration in fish fed the different carotenoid diets, but that the changes were very subtle. It seems likely that ultraviolet reflectance in conjunction with red colouration is used by cherry barbs in making mate choice decisions. This study has demonstrated that very subtle changes in colour morphology due to consumption of carotenoid diets still impact colour-associated behaviours. There is a need for further research into the effects of a wider range of diet ingredients fed to companion fish, to examine whether they may alter colouration, the impacts these may have on fish behaviours and the underlying mechanisms. Furthermore, different species are likely to alter their colour and/or colour-based behaviours differently which may also impact interactions seen in multi-species assemblages normally found in home aquaria.

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711 Table 1: Nutritional content and carotenoid concentrations in negative
712 control, 20 ppm synthetic astaxanthin (AX), 20 ppm and 40 ppm
713 natural AX diets. Concentrations expressed as mg kg^{-1}. Analyses
714 conducted on 3 g samples of each diet. Limit of quantification (LOQ)
715 was 0.03 mg kg^{-1}.

Proximate composition (%)	**Negative control**	**20 ppm synthetic-AX**	**20 ppm natural-AX**	**40 ppm natural-AX**
Protein	32.5	32.6	31.7	32.8
Total lipid	9.8	9.9	10.2	9.9
Moisture	6.8	7.6	7.7	6.9
Ash	11.3	11.0	10.7	11.2
Carotenoid				
Astaxanthin	<LOQ	19.98	22.1	44.07
Canthaxanthin	<LOQ	<LOQ	3.45	6.84
Astacene	<LOQ	0.58	-	-
Lutein	<LOQ	<LOQ	<LOQ	<LOQ
Beta-carotene	-	-	<LOQ	<LOQ
Echinone	-	-	<LOQ	<LOQ
Cis-echinone	-	-	<LOQ	<LOQ
3-hydroxyechinone	-	-	0.22	0.45
Adonirubin	-	-	11.21	22.97
Asteroidenone	-	-	0.25	0.38
Adonixanthin	-	-	1.81	3.55
Total carotenoids	<LOQ	20.56	39.04	78.26

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723 Table 2: Analyses (one-way ANOVA) of the effects of diet treatment on the change in hue distribution over a 12 week period in various body areas in male
724 and female cherry barbs (n= 24 fish per diet treatment).

	Diet		**Negative control**	**20 ppm synthetic-AX**	**20 ppm natural-AX**	**40 ppm natural-AX**
Male fish	**$F_{3,67}$**	***P***	**Mean (lower, upper bound 95% confidence interval)**			
Whole body	$_{3,15.65}$=2.18	0.13	-0.008(-0.011, -0.004)	-0.012(-0.016, -0.009)	-0.011(-0.015, -0.007)	-0.007(-0.011, -0.004)
Caudal fin	$_{3,15.43}$=2.47	0.10	-0.012(-0.015, -0.008)	-0.016(-0.02, -0.013)	-0.015(-0.019, -0.011)	-0.009(-0.13, -0.005)
Anal fin	$_{3,15.82}$=3.22	**0.05**	-0.007(-0.011, -0.004)	-0.013(-0.011, -0.004)	-0.01(-0.014, -0.007)	-0.008(-0.012, -0.004)
Anterior dorsal area	$_{3,16.43}$=3.67	**0.03**	-0.006(-0.01, -0.002)	-0.01(-0.014, -0.006)	-0.011(-0.016, -0.007)	-0.006(-0.011, -0.002)
Female fish	**$F_{3,65}$**	***P***				
Whole body	$_{3,17.03}$=1.67	0.21	-0.002(-0.009, 0.005)	0.00(-0.009, 0.008)	0.004(-0.003, 0.011)	-0.004(-0.011, 0.002)
Caudal fin	$_{3,19.99}$=1.79	0.18	0.005(-0.004, 0.014)	0.00(-0.011, 0.012)	0.01(0.001, 0.02)	0.006(-0.004, 0.015)
Anal fin	$_{3,17.74}$=2.16	0.13	-0.004(-0.009, 0.001)	-0.001(-0.008, 0.006)	0.00(-0.005, 0.005)	-0.006(-0.012, -0.001)
Anterior dorsal area	$_{3,15.74}$=1.94	0.17	0.001(-0.004, 0.006)	0.00(-0.007, 0.006)	0.008(0.003, 0.013)	-0.004(-0.009, 0.001)

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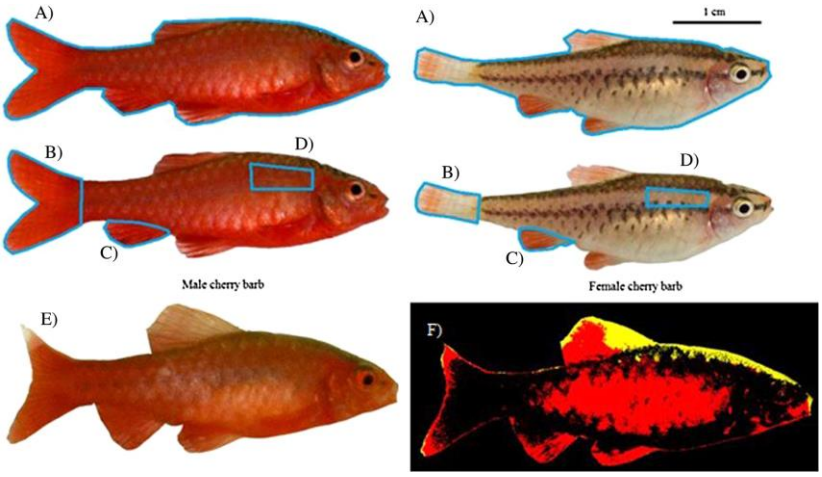


Figure 1: Male and female cherry barb colouration was analysed across the A) whole body, B) caudal fin, C) anal fin and D) anterior dorsal area. Representation of male cherry barb whole body image E) before and F) after %R and %Y calculations. Pixels within an image that fit predefined red and yellow criteria are coloured accordingly and a percentage is automatically calculated. Pixels which do not meet red or yellow criteria are coloured black. The white background of the image is recognised as not being part of the fish and is discounted from percentage calculations.

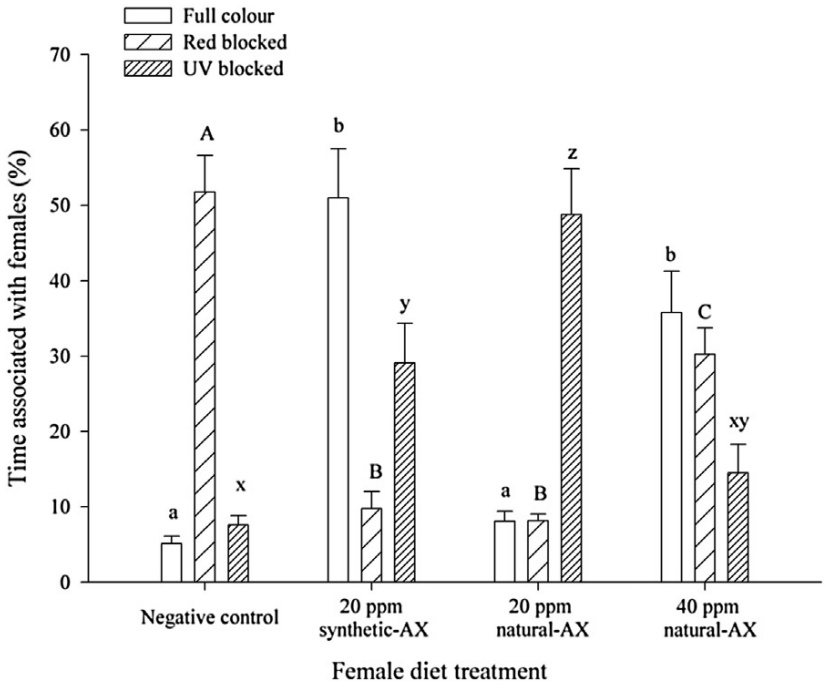
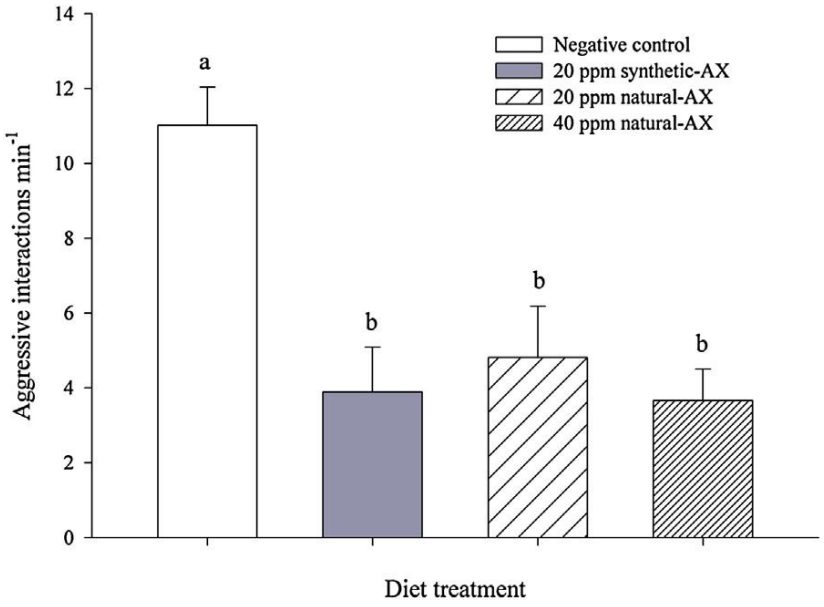


Figure 2: Mean (±S.E.M.) percentage of time male fish spent associated with females from different diet treatments (negative control, 20 ppm synthetic-AX, 20 ppm and 40 ppm natural-AX diets) under different lighting conditions (full colour spectrum lighting, red light blocked and UV light blocked) (n= 9 fish per diet treatment). Letters indicate homogenous groups between diet treatments within lighting treatments at the 5% significance level (Tukey's HSD).



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749 Figure 3: The mean (±S.E.M.) number of aggressive interactions
750 performed per minute to a mirror image by male fish from different
751 diet treatments (n= 9 fish). Letters indicate homogenous groups at the
752 5% significance level (Tukey's HSD).